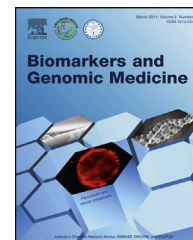




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REVIEW ARTICLE

Biomarkers in fetal alcohol syndrome

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Received 21 October 2013; received in revised form 6 January 2014; accepted 22 January 2014
Available online 6 March 2014

KEYWORDS

biomarkers;
early diagnosis;
fetal alcohol
syndrome;
prognosis

Abstract Ethanol consumption during pregnancy is a widespread problem and is increasing globally among young women. Development of biomarkers of fetal alcohol syndrome (FAS), which can identify children at risk, may lead to interventions earlier in life. In addition, animal models of fetal alcohol spectrum disorders can help in novel biomarker discovery. Biomarkers of fetal alcohol spectrum disorders include classical biomarkers of alcohol-induced pathology (mean corpuscular volume, γ -glutamyl transferase, aspartate aminotransferase, and alanine aminotransferase), acetaldehyde-derived conjugates, and derivatives of nonoxidative ethanol metabolism (fatty acid ethyl esters, ethyl glucuronide, ethyl sulfate, and phosphatidyl ethanol). Because ethanol and acetaldehyde levels can be measured in blood, urine, and sweat a few hours after ethanol intake, these can be used to detect recent ethanol exposure. Magnetic resonance spectroscopic (MRS) biomarkers include N-acetyl aspartate, an indicator of neuronal density; choline, a precursor of the neurotransmitter; acetyl choline, implicated in learning and memory and in the synthesis of glycerophosphocholine (involved in membrane synthesis); and glutamate that is reduced in FAS. Glutamate is a precursor for the synthesis of γ -amino butyric acid, and creatine is required for high-energy phosphate synthesis. Furthermore, reduced brain-derived neurotrophic factor, somatostatin, complexin, taurine, glutathione, myoinositol, leptin, and increased insulin-like growth factor and N-methyl D-aspartic acid receptor toxicity are observed in FAS. Impaired methionine–homocysteine cycle may also have deleterious effects on protein, DNA, and histone methylation in FAS. In addition to meconium fatty acid ethyl esters, magnetic resonance imaging, positron emission tomography, and single-photon-emission computerized tomography facilitate an earlier diagnosis of less alcohol-related disabilities that cannot be confirmed in the absence of a maternal drinking history. Brain volume, cortical volume, and cortical surface area are also reduced following prenatal exposure to ethanol. Hence, discovery of novel biomarkers is needed to define behavioral, physical, and genetic factors for better clinical management of FAS.

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Introduction

Alcohol and several other drugs are frequently abused alone or in combination across the globe.¹ Ethanol is the most widely used drug of abuse and a teratogen whose consumption among women of childbearing age is increasing gradually. Fetal alcohol spectrum disorder (FASD) is a developmental disorder that affects up to 0.2% of births. Because both fetal alcohol syndrome (FAS) and fetal alcohol effect represent preventable causes of mental retardation and birth defects, detection of alcohol abuse during pregnancy is essential to avoid deleterious consequences on fetal brain development. A considerable amount of work is required to extend the knowledge on fetal alcohol effects among women of childbearing age. Moreover, awareness and training among health care professionals may play significant roles in the early diagnosis of these devastating conditions.

Alcohol consumption during the gestational period causes fetal alcohol exposure and is associated with the onset of FASD, including FAS. FASD and FAS can lead to physical, cognitive, and behavioral disabilities, whose early diagnosis is important to accomplish primary prevention with total abstinence from alcohol during pregnancy and secondary prevention in newborns and children with regular follow-ups to reduce the risk of secondary consequences. In recent years, efforts have been made to understand molecular mechanisms of FAS and identify biological and diagnostic tools, such as biomarkers in neonatal meconium and magnetic resonance imaging (MRI). However, further studies are needed to extend the knowledge on fetal effects of ethanol, and multidisciplinary approaches are necessary to raise awareness among women of childbearing age about the dangers of consuming even a small amount of alcohol during pregnancy.² Deleterious consequences of FAS are well established; as a leading cause of intellectual impairments, it has social and public health impact as FAS is associated with several neurobehavioral deficits.³ FAS is the most serious consequence of prenatal ethanol exposure, and individuals who do not meet diagnostic criteria for FAS are also influenced by gestational ethanol exposure. The term FASD is used to cover a spectrum of effects that includes FAS as well as alcohol-related neurodevelopmental disorder and alcohol-related birth defects. The term alcohol-related neurodevelopmental disorder refers to a condition in which individuals, after heavy prenatal alcohol exposure, exhibit neurobehavioral deficits without meeting the physical criteria of FAS.^{4,5} Clinical identification of this group is difficult because it does not exhibit typical physical features of FAS, and physiological biomarkers of gestational alcohol exposure have limitations. Hence, determination of a profile, based on the neurobehavioral effects of prenatal alcohol exposure, would allow accurate identification of FAS and affected individuals. The development of such a profile is aimed at identifying and characterizing those who are affected by prenatal alcohol exposure, and not only those who have been exposed to alcohol prenatally.^{6,7} Acquiring information about drinking during pregnancy is one of the most challenging areas of the study on FASD, because these children are difficult to differentially

diagnose, treat, and confirm whether or not their mothers consumed alcohol during pregnancy. Currently available screening questionnaires to diagnose and confirm FASD are unreliable, and biomarkers are insensitive for pregnant women. Hence, there is a dire need to discover novel biomarkers with better sensitivity and specificity for detecting even moderate drinking during pregnancy. Gestational alcohol consumption is associated with the onset of FASD (including FAS), which can lead to physical, cognitive, and neurobehavioral impairments. Hence, an early diagnosis of FAS is extremely important for primary prevention with total abstinence from alcohol during pregnancy and for secondary prevention to reduce the risk in newborns and children during later life.

The exact molecular mechanism underlying fetal developmental defects caused by maternal ethanol consumption remains enigmatic. Unfortunately, the diagnosis of FAS and fetal alcohol effect is made after birth, when alcohol damage has become irreversible and permanent. Laboratory diagnosis can help prevent this damage and make a valuable contribution to the assessment of prenatal alcohol abuse. Especially, clinical utility of blood/breath alcohol, γ -glutamyl transferase (GGT), mean corpuscular volume (MCV), and carbohydrate-deficient transferrin (CDT) in pregnancy is remarkable. Although none of these biomarkers has adequate sensitivity and specificity, their diagnostic accuracy increases when estimated as a panel, particularly while detecting alcohol abuse in pregnancy where the presence of several positive biomarkers can be correlated in the presence of alcohol-related fetal defects.⁸

This article briefly reviews the recent literature on biomarkers as maternal risk factors for FASD and emphasizes that maternal risk is multidimensional, including factors related to quantity, frequency, timing of alcohol abuse, maternal age, number of pregnancies, mother's frequency of child birth, body size, body mass index, nutritional status, socioeconomic status, metabolism, religion, spirituality, depression, drug abuse, and social relationships. Furthermore, a brief description of various biomarkers is provided as a basic guideline for the clinical management of FAS during the early stages. Although the first trimester is considered to be the most vulnerable period, it is now realized that intrauterine exposure to ethanol may cause fetal damage throughout the entire gestational period. Based on the data obtained from the National Epidemiologic Survey on Alcohol and Related Conditions, Falk et al^{9,10} reported that 21.7% of the sampled population abused both alcohol and tobacco and 5.6% abused alcohol along with other drugs. Among women aged 18–24 years, the rates were 25.5% and 12.5%, respectively.

Individually, alcohol, tobacco, and illicit drugs (cocaine or amphetamine) are harmful to the developing fetus. Hence, determining the harm resulting from multiple drug abuse during pregnancy is a challenging task. Unpredictable interactive effects of the drugs abused simultaneously have long-term consequences on the child's health and development. Intrauterine ethanol-exposed adolescents are highly prone to developing drug dependence, and ethanol neurotoxicity is augmented by nicotine in these individuals.

By performing micro-positron emission tomography imaging with ^{18}F -DOPA and ^{18}F -dG on cocaine and methamphetamine (METH)-intoxicated C57BL/6J mice, we discovered that these illicit drugs of abuse cause a reduction in the striatal dopaminergic neurotransmission. Uptake of the striatal ^{18}F -DOPA and myocardial ^{18}F -dG was further reduced when cocaine and METH were administered along with ethanol, suggesting that alcohol accentuates cocaine and METH neurotoxicity and may be considered as the gateway to multiple drug abuse.¹¹ More deleterious consequences in learning, intelligence, memory, and delayed sensory-motor learning have been noticed among children whose mothers consumed alcohol during pregnancy. Particularly, the use of a combination of alcohol and cocaine is more harmful than that of each drug individually because of the formation of the highly toxic metabolite cocaethylene.^{12,13} Hence, FAS must be a diagnosis of exclusion, and has to be differentiated from conditions caused by other embryotoxic agents, such as environmental neurotoxin, domoic acid, malnutrition, zinc deficiency, and genetic syndromes, which share similar morphological features. In fact, FAS is not a single entity but represents the most severe form of a spectrum of disorders, including characteristic craniofacial alterations, stunted growth, and behavioral abnormalities caused by gene–environment interactions in addition to ethanol. There is no doubt that alcohol induces deleterious effects on the developing fetus, and the severity of fetal damage depends on the strength of alcohol and the frequency of its consumption during pregnancy. Depending on the frequency of alcohol consumption during pregnancy and the concentration of alcohol, still birth, preeclampsia, abortion, and fatality have been noticed to occur. In addition to FAS, maternal alcohol consumption during pregnancy may cause Trout syndrome, sudden infant death syndrome, and autism. Various neurotransmitters and their receptors may be modified and/or impaired in FAS. In particular, dopaminergic, cholinergic, γ -amino butyric acid (GABA)-ergic, glutamatergic, and serotonergic systems are impaired in FAS victims. This suggests that alcohol consumption should be absolutely prevented at any stage of pregnancy, as it may seriously affect the synaptic neurotransmission in some brain regions, which is required for normal learning, intelligence, memory, and behavior.

Definition of a biomarker

A biomarker is a distinct biochemical, genetic, or molecular characteristic or substance that serves as an indicator of a particular biological condition or process. According to Strimbu and Tavel,¹⁴ the term “biomarker” refers to a broad subcategory of medical signs, that is, an objective indication of the medical state observed from outside the patient, which can be measured accurately and reproducibly. Peterson¹⁵ provided definitions of state and trait biomarkers. In brief, a “state biomarker” provides information about recent drinking activity, whereas a “trait biomarker” provides information about a person’s genetic predisposition toward alcohol dependence. In general, GGT, alanine aminotransferase, aspartate aminotransferase (AST), CDT, N-acetyl- β -hexosaminidase, whole blood-associated acet-aldehyde, MCV, apolipoprotein J, 5-hydroxytryptophol (5-

HTOL), fatty acid ethyl esters (FAEEs), and ethyl glucuronide (EtG) are used as state biomarkers,^{16–18} whereas adenyl cyclase activity, GABA, dopamine, β -endorphin, and serotonin are used as trait biomarkers to determine the acute or chronic deleterious effects of fetal alcohol abuse.^{19–21}

Ideal biomarker of FAS

An ideal biomarker for detecting alcohol abuse should have the following characteristics: (1) the capacity to detect low-to-moderate levels of drinking over extended periods of time; (2) a high probability of detecting drinking during pregnancy (“sensitivity”); (3) a low rate of false-positive results (“specificity”); (4) easy accessibility of biological sample by a minimally invasive clinically acceptable procedure; (5) little or no sample preparation requirement; (6) a simple, economical analytical procedure of detection; and (7) ability to provide rapid results. Unfortunately, none of the presently available biomarkers adequately satisfies more than one or two of these criteria.

Clinical consequences of intrauterine alcohol

Depending on the strength of alcohol and the frequency of its consumption during pregnancy, still birth, preeclampsia, abortion, and fatality have been reported. In addition to FAS, fetal alcohol can induce Trout syndrome, sudden infant death syndrome, and autism. Adverse clinical consequences of intrauterine alcohol exposure to a developing fetus are illustrated in Fig. 1.

Classification of FAS biomarkers

In general, FAS biomarkers can be classified as *in vitro* and *in vivo* biomarkers, which can further be subdivided into clinical, molecular, omic, and imaging biomarkers. At earlier stages, cord blood, meconium, and urinary biomarkers can facilitate the assessment of deleterious consequences of FAS. In general, FAS biomarkers can be divided into the following categories: (1) clinical; (2) molecular; (3) omic; (4) imaging; (5) meconium; (6) cord blood; (7) placental; (8) plasma; (9) urinary; and (10) meconium biomarkers. We have described primarily eight FAS biomarkers, including clinical, molecular, omic, imaging, meconium, cord blood, anatomical, and neuro-behavioral biomarkers, which can facilitate early FAS diagnosis, as illustrated in Fig. 2.

Temporal window of FAS biomarkers

The concentration of the FAS biomarkers depends on the severity and duration of maternal ethanol abuse. Usually <1 drink/day is considered low, 2 drinks/day moderate, and >14 drinks/week severe. Although several biomarkers have been proposed for the early diagnosis and accurate clinical evaluation of FAS, their estimation and quantitation for the differential diagnosis has a critical temporal window. For example, within hours of birth, breath, blood, and urine alcohol levels can be estimated to assess the

Clinical Consequences of intrauterine Alcohol

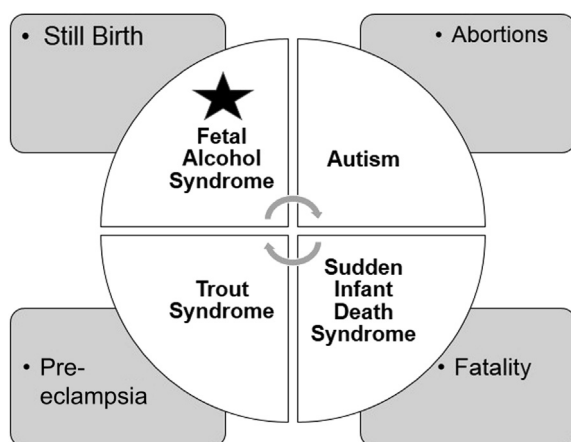


Figure 1 Clinical consequences of intrauterine alcohol. This diagram illustrates clinical consequences of intrauterine alcohol exposure to a developing fetus. Alcohol induces deleterious effects on the developing fetus, and the severity of fetal damage depends on the strength and frequency of alcohol consumption during pregnancy. In general, still birth, pre-eclampsia, abortion, and fatality have been reported. Fetal alcohol can also induce fetal alcohol syndrome, Trout syndrome, sudden infant death syndrome, and autism.

severity of maternal alcohol abuse. Urinary EtG can be detected within the first 5 days of delivery, whereas CDT and phosphatidyl ethanolamine (PEth) can be detected within <3 weeks. GGT and MCV can be detected within <3 weeks, and hair analysis of FAEEs, EtG, and PEth can be performed within <3 months. Ideally, weeks and months are the target areas for the development of novel biomarkers of FAS. Within hours of delivery, the level of breath, blood, and urine alcohol, even if low, can be detected. Moderate alcohol consumption may be detected by estimating CDT and PEth. Urinary ethanol and EtG can be estimated within weeks, whereas GGT, MCV, meconium FAEE, hair analysis of FAEE, EtG, and PEth can be detected within months in the hair, plasma, and meconium samples, as illustrated in Fig. 3. Furthermore, several *in vitro* biomarkers of FAS have been introduced, including MCV, alanine aminotransferase, FAEE, GGT, acetaldehyde-derived conjugates, EtG, ethyl sulfate (EtS), AST, derivatives of nonoxidative ethanol metabolism, and PEth to facilitate early FAS diagnosis and/or treatment. Direct maternal biomarkers include GGT, AST, alanine aminotransferase, MCV, CDT, and the ratio of (5-hydroxy tryptophol/5-hydroxy indole acetic acid) have also been estimated.

Trait versus state biomarkers of FAS

Trait biomarkers include platelet adenyl cyclase activity and increased β -endorphin responsiveness. State biomarkers are of two types, namely indirect and direct biomarkers, which can be subdivided into spot or short-

Biomarkers of FAS

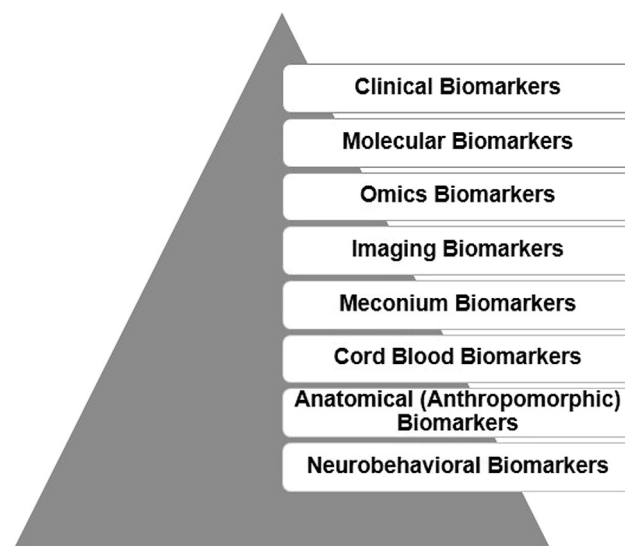


Figure 2 Biomarkers of FAS. FAS biomarkers can be divided into the following eight major types: (1) clinical biomarkers; (2) molecular biomarkers; (3) omic biomarkers; (4) imaging biomarkers; (5) meconium biomarkers; (6) cord blood biomarkers; (7) anatomical biomarkers; and (8) neurobehavioral biomarkers. FAS = fetal alcohol syndrome.

term abuse and chronic long-term abuse. For spot or short-term use, ethanol in blood, breath, and urine; EtG in urine and blood; FAEE in serum; PEth in blood; EtS and ethyl phosphate in urine and blood; and β -carboline in urine or blood can be estimated. For chronic long-term alcohol abuse, FAEE in hair and meconium, EtG in hair and meconium, cocoethylene in hair, acetaldehyde adducts of proteins in erythrocytes in hair, and β -carboline in hair may be estimated. Direct biomarkers can be subdivided into spot or short-term biomarkers (5-HTOL/5-HIAA) in urine. For chronic or long-term use, GGT in urine, alanine aminotransferase and AST in serum, MCV in blood, CDT in urine, sialic acid in serum, sialic acid index of serum apolipoproteins, β -hexosaminidase in the serum, methanol in blood, and dolichol in blood and urine can be estimated for the clinical assessment of FAS, as illustrated in Fig. 4.

Clinical biomarkers of FAS

Chronic intake of ethanol during pregnancy can primarily cause FAS, autism, and sudden infant death syndrome. Depending on the amount of alcohol consumed and the frequency of consumption during early stages of pregnancy, still birth, abortion, or preeclampsia may occur. Clinical biomarkers of FAS can be subdivided into anatomical biomarkers, developmental biomarkers, neurological biomarkers, delayed motor learning, and impaired handwriting. In general, anatomical biomarkers include facial abnormalities, micrognathia, small-size lips, reduced frontal lobe development, and reduced eye blinking. In particular, neurological biomarkers of FAS

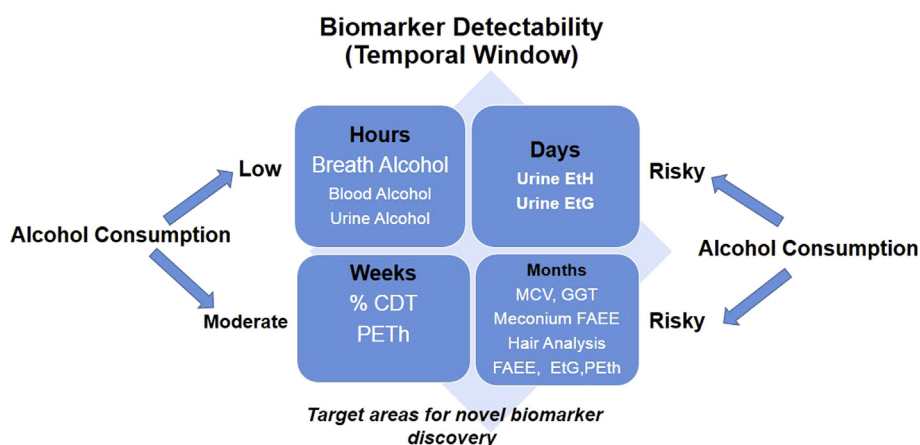


Figure 3 Temporal window of biomarkers detectability. Different FAS biomarkers can be detected within hours, days, weeks, and months, depending on the amount of alcohol consumed during pregnancy. Within hours of delivery, breath, blood, and urine alcohol levels can be detected even at a low concentration of alcohol; moderate alcohol consumption may be detected by estimating % CDT and PEth. Higher concentration of alcohol may have deleterious consequences. Urinary ethanol and ethylene glucuronide can be estimated within weeks, whereas MCV, GGT, meconium FAEE, and hair analysis of FAEE, ethylene glucuronide, and phosphatidyl ethanolamine can be detected within months in FAS victims. CDT = carbohydrate-deficient transferrin; EtG = ethyl glucuronide; FAEE = fatty acid ethyl ester; FAS = fetal alcohol syndrome; GGT = gamma glutamyl transferase; MCV = mean corpuscular volume; PEth = phosphatidyl ethanolamine.

include mental retardation, slow learning, reduced intelligence, reduced memory retention, and abnormal aggressive behavior.

Morphological biomarkers of FAS

Not all women who consume alcohol during pregnancy have children with FASD, as genetic factors, nutritional status, and body mass index of the mother may also play significant roles in ethanol teratogenesis. The lack of a convincing molecular biomarker has hindered FASD research and treatment. However, FASD comprises severe cognitive and structural birth defects including short stature, small head, cleft lip/palate, small jaw, wide-set eyes, dental abnormalities, and digit abnormalities. Interestingly, mutations in an inwardly rectifying potassium channel, Kir2.1, cause similar birth defects as noticed in FASD. In other words, FASD phenocopies the traits conveyed by Kir2.1 mutations. Recently, Bates²² reported that alcohol targets Kir2.1 to cause the birth defects associated with FASD. Clinical, genetic, biochemical, electrophysiological, and molecular evidence also identified Kir2.1 as a molecular target for FASD development and therapeutic treatment. Recently, Downing et al²³ examined gene expression in embryos and placentae from C57BL/6J (B6) and DBA/2J (D2) mice following prenatal alcohol exposure. B6 fetuses were susceptible to morphological malformations, whereas D2 fetuses were relatively resistant. These investigators mated male and female B6 and D2 mice to produce four embryonic genotypes: true-bred B6B6, D2D2, reciprocal B6D2, and D2B6. On Gestational Day 9, dams were intubated with 5.8 g/kg ethanol, an isocaloric amount of maltose dextrin, or nothing. Four hours later, dams were sacrificed, and embryos and placentae were harvested. RNA was extracted and hybridized to an Affymetrix Mouse Genome microarray chip. Several genes were differentially expressed in B6 and D2 regardless of

treatment, including genes involved in polysaccharide binding and mitosis. Altered expression of genes involved in methylation, chromatin remodeling, protein synthesis, and mRNA splicing was observed. Altered gene expression in B6 included those involved in mRNA splicing, transcription, and translation. The lack of strain-specific effects in D2 suggests that only a few genes induce resistance.

Neuroimaging studies on individuals with FASD have provided links between prenatal alcohol exposure and morphological deficits. Yang and colleagues^{24,25} collected the structural MRI data from 69 children and adolescents with FASD and 58 controls, to detect cortical thickness changes in FASD and their associations with facial dysmorphology. Individuals with FASD showed thicker cortices than controls in frontal, temporal, and parietal regions. Increased inferior frontal thickness was correlated with reduced palpebral fissure length. This study suggested the increase in cortical thickness as a biomarker for impaired brain development in FASD. The associations between cortical thickness and dysmorphic measures suggested that the severity of brain anomalies may be reflected on the face. Ocular defects have been reported in about 90% of children with FAS, including microphthalmia, loss of neurons in the retinal ganglion cell (RGC) layer, optic nerve hypoplasia, and dysmyelination. Estimations of neurons in the ganglionic cell layer (GCL) and dorsolateral geniculate nucleus (dLGN), and analysis of RGC morphology were carried out in transgenic mice exposed to ethanol during early postnatal life. Dursun and colleagues²⁶ performed this study in male and female transgenic mice expressing yellow fluorescent protein under the control of thymus cell antigen 1 (Thy-1) regulator using C57 background. Ethanol (3 g/kg/day) was administered to pups by intragastric intubation throughout postnatal Days 3–20. Blood alcohol concentration (BAC) was measured on postnatal Day 3, Day 10, and Day 20 after the second intubation. Numbers of neurons in the GCL and dLGN were quantified on postnatal Day 20

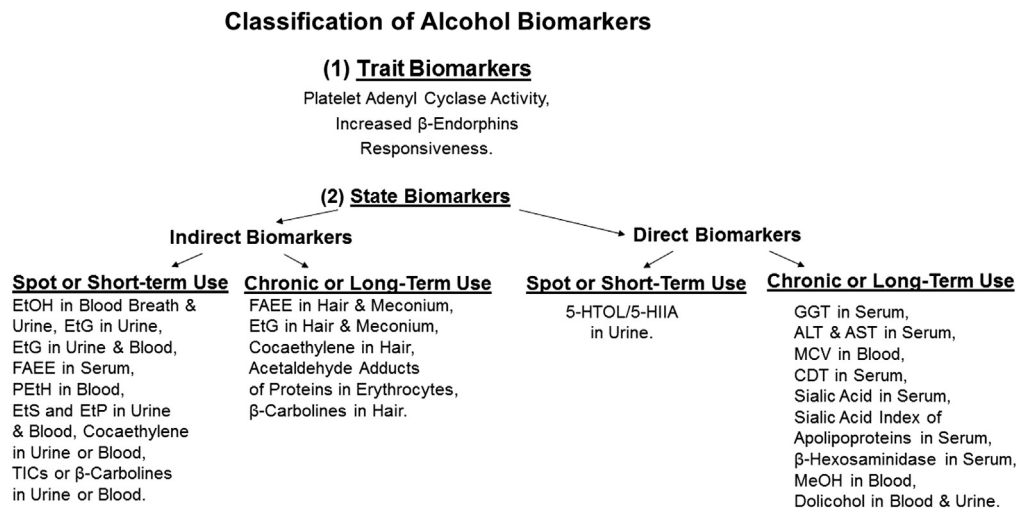


Figure 4 Classification of FAS biomarkers. In general, FAS biomarkers may be divided in two broad categories: (1) trait and (2) state biomarkers. Trait biomarkers include platelet adenyl cyclase activity and increased β -endorphin responsiveness. State biomarkers are of two types: indirect and direct biomarkers. Indirect and direct biomarkers can be subdivided into spot or short-term use and chronic long-term use. For spot or short-term use, we can estimate ethanol in blood breath and urine, EtG in urine and blood, FAEE in serum, PEth in blood, EtS and EtP in urine and blood, and TICs or β -carboline in urine or blood. For chronic long-term use, we can estimate FAEE in hair and meconium, EtG in hair and meconium, cocaethylene in hair, acetaldehyde adducts of proteins in erythrocytes in hair, and β -carboline in hair. Direct biomarkers can be subdivided into spot or short-term use (5-HTOL/5-HIAA) in urine. For chronic or long-term use, GGT in urine, ALT and AST in serum, MCV in blood, CDT in urine, sialic acid in serum, sialic acid index of serum apolipoproteins, β -hexosaminidase in the serum, methanol in blood, and dolichol in blood and urine can be estimated. ALT = alanine aminotransferase; AST = aspartate aminotransferase; CDT = carbohydrate-deficient transferrin; EtG = ethyl glucuronide; EtP = ethyl phosphate; EtS = ethyl sulfate; FAEE = fatty acid ethyl ester; FAS = fetal alcohol syndrome; GGT = gamma glutamyl transferase; MCV = mean corpuscular volume; PEth = phosphatidyl ethanolamine; 5-HTOL = 5-hydroxytryptophol; TICs = sudden, rapid, nonrhythmic movements.

using stereological procedures. Ethanol exposure in mice altered RGC morphology and decreased the number of neurons in the GCL and dLGN. The soma was significantly reduced and dendritic tortuosity increased in RGCs. A decrease in total dendritic field area and an increase in the branch angle were also observed. Dendritic elongation and a decrease in the spine density were observed in the IC group, as compared to ethanol-exposed and control subjects. No significant effects were observed on the total retinal area, suggesting that an early postnatal ethanol exposure affects the development of visual system by reducing the number of neurons in the GCL and dLGN, and by altering RGC morphology.

The time of onset of FAS is difficult to determine because of the challenge associated with *in vivo* studies of the peri-implantation stage of embryonic development. Because embryonic stem cells (ESCs) are derived from blastocyst-stage embryos, these cells have been used as a model for investigating early embryonic development. VandeVoort and coinvestigators²⁷ performed an *in vitro* study on ESC lines (ORMES-6 and ORMES-7) of rhesus monkeys treated with ethanol, estradiol, or acetaldehyde with or without estradiol for 4 weeks. Although control ESCs remained unchanged, abnormal morphology in the ethanol- and acetaldehyde-treated groups was observed prior to 2 weeks of treatment. Immunofluorescence staining of pluripotency markers (TRA-1-81 and alkaline phosphatase) indicated a loss of ESC pluripotency in the 1.0% ethanol group. The ORMES-7 cell line was more sensitive to ethanol

than the ORMES-6 cell line. Estradiol increased sensitivity to ethanol in both the ORMES-6 and the ORMES-7 cell line. This study suggested that the effects of ethanol may be mediated through metabolic pathways regulating acetaldehyde formation; however, estradiol-induced increase in sensitivity to ethanol remains unknown.

In mice, ethanol exposure on gestational Day 7 results in ventromedial forebrain deficits along with facial anomalies characteristic of FAS. Godin and coworkers²⁸ explored ethanol's teratogenic effect on the ventromedial forebrain in a mouse model. Mated C57Bl/6J mice were injected with 2.9 g/kg ethanol or saline twice, at a 4-hour interval, on their 7th day of pregnancy. On gestational Day 12.5, Day 13, and Day 17, control and ethanol-exposed specimens were collected to assess morphological changes. Cerebral hemispheres (forebrains) of ethanol-exposed embryos were too close in proximity or rostrally united, with enlarged foramina of Monroe or united lateral ventricles, and hippocampal and ventromedial forebrain deficiency. On gestational Day 12.5, in control and ethanol-exposed embryos, *in situ* hybridization confirmed the selective loss of ventromedial region. Immunohistochemical labeling of oligodendrocyte progenitors with Olig2, a transcription factor necessary for their specification and of GABA, showed reductions. Somatostatin-expressing interneurons were dysmorphic in the ethanol-exposed fetuses. In addition, facial abnormalities, micrognathia, small lip size, reduced frontal lobe development, and reduced eye blinking were observed in the progeny with fetal alcohol exposure.

Neurological biomarkers of FAS

In general, neurological biomarkers of FAS include mental retardation, reduced memory retention, slow learning, abnormal behavior, reduced intelligence, and aggressive behavior. Multicenter neuroimaging trials, consortia, and collaborations enabled the acquisition of data that can be used to predict clinical outcomes as well as treatment options for FAS.²⁹ Symptoms of FAS include neurological and immunological dysfunctions linked to cell reduction. Hao and colleagues³⁰ exposed CD34+ human fetal liver hematopoietic stem cells (HSCs) and CD133+/nestin+ human neural stem cells (NSCs) to 0.1–10 mM ethanol, which induced deleterious effects on NSCs but had no significant effects on HSCs. The colony-forming ability of NSCs was completely inhibited by 5 mM ethanol treatment, whereas HSCs were unaffected by even 20 mM ethanol, suggesting selective sensitivity of NSCs to ethanol. Protein kinase C (PKC) isoenzyme expression was also differentially affected by ethanol in NSCs and HSCs. These observations also explain the lack of hematopoietic problems in FAS.

Molecular biomarkers of FAS

Lee and colleagues³¹ performed differential mRNA analyses to characterize the etiology and discover potential molecular biomarkers for FAS. Out of approximately 1080 mRNA transcripts in mouse embryos, the levels of three mRNAs were altered. Two of these mRNAs (1 novel and 1 encoding heat shock protein-47) were also modulated by 3-methylcholanthrene. The third mRNA, encoding α -tropomyosin, was specifically upregulated by ethanol. The level of α -tropomyosin, a brain-specific isoform, was elevated in the ethanol-exposed embryos. Several well-known medications, such as aspirin, cimetidine, and ranitidine, interact with alcohol metabolism, leading to increased BAC.^{32–34} Given that BAC is a reliable predictor of the severity of alcohol-mediated brain injury in preclinical studies, any drug that interferes with alcohol metabolism and results in an increase in BAC may be a cofactor in increasing alcohol-mediated damage.^{35,36} Johnson and colleagues³⁷ showed that cigarette smoking reduces peak BAC in humans. Preclinical studies have supported these findings.^{38,39} A decrease in BAC in the presence of nicotine may suggest less harmful effects of alcohol; however, if someone desires to experience the “high” from alcohol or drink to the point of inebriation, this decrease in BAC may promote additional alcohol abuse, which may lead to an accumulation of acetaldehyde, a toxic metabolite that may cause further damage to the developing fetus. Recently, Ismail and colleagues⁴⁰ reviewed the teratogenic effects of alcohol, strategies for detecting maternal alcohol consumption, identification of fetal biomarkers, and prevention procedures for FAS. Furthermore, Roehsig and coworkers⁴¹ developed a method for the determination of eight FAEEs (ethyl laurate, ethyl myristate, ethyl palmitate, ethyl palmitoleate, ethyl stearate, ethyl oleate, ethyl linoleate, and ethyl arachidonate) in meconium samples. FAEEs could be detected in alcohol-exposed newborns. These could also be detected in some nonexposed newborns, although the concentrations were lower than those measured in exposed

cases. Dutta and colleagues⁴² used amniotic fluid for biomarker analyses to discriminate between FAS-positive and FAS-negative pregnancies in mice. B6J and B6N litters were treated with alcohol or saline on Day 8 of gestation. Preliminary characterization revealed reduced peaks to α -fetoprotein, supporting clinical observations. Furthermore, Través and coworkers⁴³ proposed apolipoprotein A-IV (apoA-IV) as a biomarker for evaluating the effects of intrauterine ethanol on the development of the intestine. These investigators estimated serum biomarkers to determine the structural integrity of the intestine. Exposure to ethanol *in utero* reduced the levels of apoA-IV in serum at birth, regardless of body weight, suggesting that circulating apoA-IV could be used as a biomarker of FAS. Barr and colleagues⁴⁴ reported that rodents that are prenatally exposed to ethanol exhibit a wide range of cognitive deficits, including impairments in memory, attention, and executive function. These investigators measured levels of presynaptic proteins complexin I and II in a rat model of prenatal ethanol exposure, as levels of these proteins are altered in cognition-related synaptic plasticity. Prenatal exposure to ethanol did not alter presynaptic proteins in the hippocampus or levels of synaptophysin; however, these rats displayed reduced levels of complexin I and II in the prefrontal cortex.

Lee and colleagues³¹ determined the molecular mechanisms underlying alcohol-induced ocular anomalies in *Xenopus* embryos, exposed to various concentrations (0.1–0.5%) of alcohol, and the subsequent effects on eye development and eye biomarker gene expression. Furthermore, Peng and coworkers⁴⁵ investigated the role of reactive oxygen and nitrogen species in FAS-associated ocular injury, using antioxidant enzymes, catalase, and peroxiredoxin 5, which were overexpressed in the blastomeres of *Xenopus* embryos. Alcohol caused microphthalmia, incomplete closure of the choroid fissure, malformation of the retina in 40% of the eyes, and reduced expression of eye biomarker genes, of which *TBX5*, *VAX2*, and *Pax6* were highly vulnerable. Overexpression of mitochondrial catalase and peroxiredoxin 5 restored the expression of these biomarkers and decreased ocular malformation. Although these antioxidant enzymes reduced the production of alcohol-induced reactive oxygen species, only peroxiredoxin 5 inhibited the formation of reactive nitrogen species, suggesting that oxidative and nitrative stresses contribute to alcohol-induced fetal ocular injury.

Meconium biomarkers of FAS

The biomarkers of FAS that can be detected from meconium include FAEE, EtG, PEth, and EtS. Their estimation can provide a reliable measure of alcohol consumption during pregnancy, as these are relatively stable and can be estimated even after 2–3 months of delivery. In addition to self-reports and questionnaires, biomarkers are of relevance in the diagnosis and treatment of alcohol abuse disorders.⁴⁶ Traditional biomarkers such as GGT or MCV are indirect biomarkers and are influenced by age, gender, and non-alcohol-related diseases, whereas EtG, EtS and PEth are direct metabolites of ethanol and hence represent useful diagnostic tools for identifying alcohol abuse more

accurately. These biomarkers remain positive in serum and urine for a considerable length of time. They are applied in routine clinical use, in emergency room settings, for providing proof of abstinence in alcohol rehabilitation programs, for detecting offenders driving under alcohol influence, in workplace testing, for assessing alcohol intake during liver transplantation, and for evaluating FAS. Due to these properties, these biomarkers may open up further avenues for prevention, interdisciplinary cooperation, diagnosis, and treatment of alcohol-related disorders. Natekar and coinvestigators⁴⁷ studied clinical cases of both cocaine and alcohol abuse, and determined hair cocaine, benzoylecgonine, Cocaethylene (CE), and FAEs. Concentrations of cocaine and benzoylecgonine were associated with increased FAE. The positive predictive value of CE was 0.66 for excessive drinking and 0.76 for chronic drinking among cocaine addicts. The negative CE value ruled out excessive alcohol consumption. Positive hair CE results had high specificity for chronic alcohol consumption among cocaine addicts. Thus, identification of CE in the hair of pregnant women who abuse cocaine can serve as a biomarker for FASD.

In the past few years, nonoxidative ethanol metabolites have received attention because of their specificity and wide time window of detection in nonconventional matrices from pregnant mothers (oral fluid and hair) and from fetuses or newborns (neonatal hair, meconium, placenta, and umbilical cord). Joya and colleagues⁴⁸ reviewed analytical procedures for the determination of these biomarkers during pregnancy and related prenatal ethanol exposure. These investigators reported that fetal alcohol disorders are preventable, but self-reported alcohol consumption could be misleading and impede effective treatment. Their study evaluated the relationship between blood PEth and alcohol abuse. Alcohol was estimated by self-report methods in 80 nonpregnant women aged 18–35 years. PEth had a positive linear association with the amount of alcohol consumed, and was detectable in 93% of individuals consuming an average of two or more drinks per day. Increasing evidence indicates that neurons and glia are direct targets of alcohol, but may also be vulnerable to molecules produced in peripheral systems as a result of alcohol exposure. Diagnostics and therapies can take advantage of these biomarkers, and these may be applicable to central nervous system pathology (CNS) in FASD.⁴⁹ A study was performed to evaluate the experience, knowledge, and confidence of Italian and Spanish neonatologists and pediatricians with respect to the diagnosis of FAS and FASD, and to evaluate awareness of maternal drinking patterns during pregnancy.⁶ A multiple-choice questionnaire was e-mailed to Italian neonatologists, and presented to Italian and Spanish pediatricians during the National Congress. The response rate was 16% (63/400) for the Italian neonatologists, whereas a total of 152 Spanish and 41 Italian pediatricians completed the questionnaire. Over 90% of the physicians declared that FAS is an identifiable syndrome, and over 60% of them identified at least one of the most important features of FAS. Although over 60% Italian and around 80% Spanish responders were aware that ethanol abuse in pregnancy is dangerous, approximately 50% Italian and 40% Spanish responders allowed women to drink a glass of wine or beer during pregnancy. Neonatologists and pediatricians rated confidence in the ability to diagnose FAS and

FASD as low. Over 50% of responders felt the need for more knowledge regarding the identification of FAS and FASD in newborns and children. However, all the responders did not feel confident about diagnosing FAS and FASD accurately, indicating the dire need for further studies on the novel discovery of FAS biomarker(s).

To study the effect of *in utero* alcohol exposure on insulin-like growth factor (IGF) and leptin during infancy and childhood, Aros and colleagues⁵⁰ identified heavily drinking pregnant women who consumed on average four or more drinks of ethanol per day (≥ 48 g/day) and assessed growth in 69 of their offspring and control group of 83 children by measuring serum IGF-I, IGF-II, IGF-binding protein 3, and leptin at 1 month and 1-, 2-, 3-, 4-, and 5-years of age. IGF-II levels increased with age in both groups, but levels were higher in ethanol-exposed children at 3-, 4-, and 5- years of age. IGF-I levels were higher at 3 years and 4 years and leptin levels were lower at 1 year and 2 years. A lower correlation between IGF-I and growth parameters suggested that exposure to ethanol during pregnancy increases IGF-I and IGF-II and decreases leptin during early childhood, and that leptin may be explored as a potential biomarker for prenatal alcohol exposure. Recent studies have shown that alcohol alters DNA methylation patterns and inhibits NSC differentiation,⁵¹ whereas Emodin prevents ethanol-induced developmental anomalies in cultured mouse fetus.⁵² Van Horn and Toga²⁹ performed a study to review multicenter neuroimaging trials and their implications for clinical treatment. These authors reported that geographic factors underlying diseased populations coupled with complementary neuroimaging research has increased neuroimaging trials and consortia. In this context, neuroimaging has become a major focus for multi-institutional research on FAS. Bakhireva and colleagues⁵³ performed a study to evaluate the feasibility and cost of Prenatal alcohol exposure (PAE) screening by measuring PEth in dried blood spot (DBS) cards. Their findings suggested that PAE screening by PEth in DBS is simpler and less expensive as compared to other screening approaches. Moreover, screening by PEth analysis from DBS cards was cost efficient. The relationship between alcohol consumption and PEth was stronger in women with recent heavy drinking. Hence, PEth is a sensitive indicator of moderate and heavy alcohol consumption, and may complement the use of self-report alcohol screens when additional objective biomarkers of alcohol abuse are desirable. However, choosing a valid cutoff concentration for PEth to differentiate various levels of alcohol consumption may not be feasible.⁵⁴

Cord blood biomarkers of FAS

Accurate confirmation of PAE is required as a diagnostic criterion for the majority of children who do not manifest the physical features associated with FAS. Cord blood biomarkers of FAS including FAEs, PEth, EtG, and EtS can be detected and estimated.

Omic biomarkers of FAS

Although several molecular and genetic biomarkers have been discovered, none of them is selective. Omic

biomarkers of FAS can be divided into proteomic, metabolomics, lipidomic, glycomic, and genomic biomarkers.

Imaging biomarkers of FAS

Imaging biomarkers of FAS include computed tomography biomarkers, positron emission tomography imaging biomarkers, MRI imaging and MR spectroscopy, and ultrasound imaging. FAS and related disorders (FASD) cause physical and mental damages to the affected individuals. Previously, histological studies in animals reported cerebral cortical abnormalities that result from prenatal ethanol exposure. Additionally, MRI identified abnormalities in the cerebral cortices of children and adolescents. However, there is still a need to bridge the gap between human MRI and animal histological studies. Leigland and colleagues⁵⁵ performed postmortem MRI experiments on rodents, during time periods relative to late human gestation through adulthood, to characterize anomalies associated with FASD throughout development. By determining how histologically identified abnormalities are manifested in MRI during the critical early time points, neuroimaging-based biomarkers of FASD can be identified at much earlier ages in humans, thus reducing the impact of these disorders. Cerebral cortical volume, thickness, and surface area were characterized by MRI in rat pups born from dams that were ethanol treated, maltose/dextrin treated, or untreated throughout the gestational period at six developmental time points (1–60 days). Brain volume, cortical volume, cortical thickness, and cortical surface area were reduced following prenatal exposure to ethanol. Cortical surface area and cortical thickness results contributed to interpreting effects of prenatal ethanol exposure on cerebral cortical development. Additionally, regional cortical thickness analyses suggested that primary sensory areas are particularly vulnerable to gestational ethanol exposure. Structural MRI measurements were in accordance with histological studies performed in animal models of FASD, suggesting that MRI is sensitive enough to detect neuroanatomical defects of fetal ethanol exposure on the development of cerebral cortex during late gestation in humans. This study provided a link between animal histological data and human MRI data. Ikonomidou and colleagues⁵⁶ reported that ethanol treatment on postnatal Day 7 (P7) causes brain cell death and can be used as a model of late gestational alcohol exposure. To investigate the long-term effects of P7 ethanol treatment on adult brain, mice received two doses of either saline or ethanol on P7 (2.5 g/kg, S.C., 2 hours apart), and were assessed as adults (P82) for brain volume and cellular architecture. MRI revealed that P7 ethanol-exposed adult mice had reduced total brain volume (4%) with reduced brain regions. Reduced frontal cortical parvalbumin immunoreactive interneurons and Cux1 + IR layer II pyramidal neurons were observed in both sexes. Biomarkers of adult hippocampal neurogenesis differed between sexes, with only ethanol-treated males showing increased doublecortin and Ki67 expression in the dentate gyrus, with increased neurogenesis compared to controls, suggesting that P7 ethanol treatment causes persistent reductions in

adult brain volume and frontal cortical neurons in both males and females. However, increased adult neurogenesis was observed in males, but not in females, which is consistent with differential adaptive responses to P7 ethanol toxicity between the sexes. Thus, 1 day of ethanol exposure is sufficient to cause persistent adult brain dysmorphology.⁵⁷

MRS biomarkers of FAS

MRS biomarkers of FAS include N-acetyl aspartate (indicator of brain regional neuronal density); choline (a precursor of the neurotransmitter); acetyl choline, implicated in learning memory and in the synthesis of glycerophosphocholine (involved in membrane synthesis); and glutamate (which is significantly reduced in FAS). Glutamate is a precursor for the synthesis of GABA (an inhibitory neurotransmitter), and creatine is required for high-energy phosphate synthesis. Reduced taurine, glutathione, myoinositol, and N-methyl D-aspartic acid receptor excitotoxicity have also been observed in the FAS. Methionine–homocysteine cycle may have serious effects on the protein, DNA, and histone methylation during FAS. Several other neurochemicals may be altered in FAS and can be estimated by magic angle spinning high-resolution MRS analysis (beyond the scope of this review). Urinary analysis of EtS can be made by high-performance liquid chromatography, in addition to the estimation of brain regional concentrations of N-acetyl aspartic acid (NAA), choline, creatine, and glutamate by MRS to confirm FAS diagnosis.

FAS and depression

It has been shown that women who drink heavily and who bear children with FASD are likely to have a history of heavy drinking in their families of origin and procreation and also in their peer groups.⁵⁸ Depression is a common complaint among mothers of children with FASD.^{59–61} Women who bear FASD children are also heavy drinkers. Mothers of FASD children in some countries use other drugs in addition to alcohol. Moreover, smoking among mothers of FASD children and drinkers may also affect the mood. We recommended tryptophan-rich to alleviate symptoms of depression as it happens due to a depletion of brain regional serotonin (5-HT).⁶² As such 5-HT cannot pass through the blood–brain barrier. However, its precursor, tryptophan, can easily pass through the blood–brain barrier, which in the presence of pyridoxine (vitamin B₆) facilitates the synthesis of brain regional 5-HT by decarboxylation of 5-hydroxy tryptophan. Hence, a diet rich in tryptophan may alleviate symptoms of depression, particularly in pregnant women consuming alcohol. Very few studies are available in this direction.

Conflicts of interest

The authors declare no conflicts of interest related to this work.

Acknowledgments

The authors express sincere thanks to Kallol Guha, President Saint James School of Medicine, Bonaire, for his encouragement and moral support.

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